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(54) Title: ARRAYS OF GLYCAN MOLECULES (GLYCOARRAYS) ON THE SURFACE OF BIOCHIPS (GLYCOCHIPS) AND USES THEREOF

(57) Abstract: The present invention relates to arrays of discrete sensing elements of glycans (glycoarrays) wherein glycans are immobilized on a solid support (glycochips). Furthermore, the present invention relates to a method of producing such glycoarrays comprising immobilization of a glycan on a preferably streptaviding coated sensing surface via biotin or a derivative thereof. In addition, the present invention relates to the use of such glycoarrays for discriminating complex biological samples, diagnosing a disease which correlates with the presence or absence of glycan binding molecules as well as to methods of identifying an organism by generation of signal pattern from a biological sample, which is indicative for the presence or absence of a particular organism. Furthermore, the present invention relates to a kit comprising the glycoarray of the invention useful in diagnostic assays.

Arrays of glycan molecules (glycoarrays) on the surface of biochips (glycochips) and uses thereof

The present invention relates to arrays of discrete sensing elements of glycans (glycoarrays) wherein glycans are immobilized on a solid support (glycochips). Furthermore, the present invention relates to a method of producing such glycoarrays comprising immobiliziation of a glycan on a preferably streptavidin coated sensing surface via biotin or a derivative thereof. In addition, the present invention relates to the use of such glycoarrays for discriminating complex biological samples, diagnosing a disease which correlates with the presence or absence of glycan binding molecules as well as to methods of identifying an organism by generation of signal pattern from a biological sample, which is indicative for the presence or absence of a particular organism. Furthermore, the present invention relates to a kit comprising the glycoarray of the invention useful in diagnostic assays.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated herein by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

Current diagnostic testing approaches used to determine the general state of health such as hemagloblin, blood pressure and the like give only limited information. And while these tests provide useful information as to the general state of health, they do not provide adequate information to identify diseases nor are they sensitive enough to detect subtle changes required for early disease detection. There is a need, therefore, to develop new assay means for the identification of disease states which provide information as to which class of ailments the patient is suffering in order to

reduce the number of specific tests which must be performed. A similar situation exists in food stuff and environmental testing.

Biochemical analyses are invaluable, routine tools in the study of biology. In order to successfully control biological processes, it is imperative that an understanding of biological interactions between various species is gained. Indeed, an understanding of biological interactions between various species is important for many varied fields of science. Many biochemical analytical methods involve immobilization of a biological binding partner of a biological molecule on a surface, exposure of the surface to a medium suspected of containing the molecule, and determination of the existence or extent of molecule coupling to the surface-immobilized sensing element. Increasing throughput demands have lead to the development of a wide range of so-called "high throughput" or HTP technologies. Initially these were based on the 96 well microtiter plate format. The well density has been sequentially increased from 96 to 384, from 384 to 1536 and now even to 6144 wells using the same footprint. The primary driving forces behind increasing the sample density were: increased throughput demands, reduced reagents usage (and therefore price) and the economies of scale.

A case in point is for example that DNA sequence libraries being produced by the Human Genome project and other genome sequencing projects required the development of an assay technology that would increase throughput not by the traditional factors of 2 or 3 but rather by 2 or 3 orders of magnitude. The technology that emerged, microarray technology, has become the work horse for HTP expression and genome analysis (Chee, Science 274 (1996), 610-614; DeRisi, Nature Genetics 14 (1996), 457-460; Schena, DNA microarrays: A practical approach, Oxford University Press, Oxford (1999)). Subsequently, a variety of microarray technologies and other HTP technologies have been developed (see companies such as Affymetrix, Incyte, Orchid, Sequenom, Lumina and Luminex). As the data from genome wide expression analysis grew into a library of differentially expressed mRNAs, and as the body of information about differentially expressed genes grew, it became evident that mRNA expression levels did not always correlate with protein expression levels (Gygi, Mol. Cell Biol. 19 (1999), 1720-1730). This lead many to realization that DNA arrays would need a protein counterpart to

confirm the relevance of an increase in mRNA expression, as well as for protein characterization. The underlying implication was that increased mRNA expression was only one part of the picture and that in order to access differentially expressed genes protein arrays would be recquired.

The term "proteome" refers to all the proteins expressed in a cell and represents tha next level of biological complexity. Protein based microarrays are currently under development in several microarray labs (Lueking, Anal. Biochem. 270 (1999), 103-111) as well as other approaches (Shevchenko, PNAS 96 (1999), 14177-14179; Gauss, Electrophoresis 20 (1999), 575-600; Link, Nature Biotechnology 17 (1999), 676-682; Pasa, JACS 121 (1999), 7949-7950). Protein based microarrays have to address a number of issues that DNA arrays did not have to deal with, for example increased demands regarding the integrity of the tertiary protein structure for retention of complex, sensitive biological activities during the preparation and use of the microarrays. Furthermore, proteins undergo numerous complex post-translation modifications making the actual number of species in the proteome far greater than in the genome. For this very reason, protein microarrays are of even greater significance as tools for analyzing the proteome.

Methods for the production of surface layers which are capable of selective biomolecular interactions and sensing surfaces produced by means of these methods and uses thereof have thus been numerously described in the prior art. However, these assays may not record diseases which are not immediately associated with the mere presence of, e.g., a polynucleotide or protein. For example, the polynucleotide based assay may not be able to distinguish between splice variants of a given gene which may lead to different proteins. Likewise, a protein recognition based assay may not be able to discriminate between certain isoforms of enzymes one of which may be the causative agent of a disease. In fact, there appears to be a variety of diseases the diagnoses of which and more importantly their causative agent has not yet become feasible due to certain known but also unknown limitations of assays presently employed.

Thus, the technical problem underlying the present invention is to provide assay means and methods that can be used in a variety of diagnostics such as health state of a subject.

The solution to this technical problem is provided by the embodiments characterized in the claims.

Accordingly, the present invention relates to an array of discrete sensing elements of glycans (glycoarray), wherein glycans of the type G-Y-X (1) are immobilized on a solid support and wherein
X is an attachment group for immobilization on the solid support;
Y is a linker group; and
G is a glycan moiety comprised of at least one or more saccharides.

The term "discrete sensing element" as used herein means elements of known chemical structure at defined positions on the surface. In particular, the term "sensing element" refers to the molecular recognition repertoire of a moiety. In this invention, this refers to the ability of a given discrete glycan or glycoconjugate bound to a solid support to bind component(s) contained in the sample. Biologically relevant molecular recognition event(s) can occur in a variety of modes. Components(s) may bind directly to the glycan moiety. In cases where the glycan itself is the target of the molecular recognition event, said glycan may act by altering the conformation, stability and the like, of the non-glycan moiety, hence altering the molecular recognition repertoire of said non-glycan moiety. Furthermore, additional component(s) may be required in order to develop the biologically relevant molecular recognition repertoire of a given glycan or glycoconjugate. A combination of any of these schemes is also possible.

The term "immobilized", used with respect to a species, refers to a condition in which the species is attached to a surface with an attractive force stronger than attractive forces that are present in the intended environment of use of the surface, and that act on the species. For example, a self assembling monolayer immobilized at a surface, the surface being used to capture a biological molecule from a fluid

medium, is attracted to the surface with a force stronger than forces acting on the glycan moiety in the fluid medium, for example solvating and turbulent forces. Immobilization may, e.g., mean covalent interaction, ionic interaction, hydrogen bonds, hydrophobic interactions and the like (see, e.g., Voet, Biochemie, VHC (1992)). In this context the term "surface" in particular refers to the outermost accessible molecular domain of any generally two-dimensional structure on a solid substrate. A surface may have steps, trenches, ridges, terraces, and the like made using etching, stamping, micromachining and the like without ceasing to be a surface.

A wide variety of immobilization techniques are available in the state of the art to efficiently and site-specifically couple glycans and glycoconjugates to a wide range of commercially available solid supports (Deutscher, ed., 1990 Guide to protein purification: Methods in enzymology, Vol. 182, Academic Press; Bickerstaff, ed., 1997, Immobilization of enzymes and cells, Humana Press). The Pierce catalog (www.piercenet.com) contains a wide range of cross-linking reagents also useful for immobilizing biomolecules to solid supports.

The term "capturing" refers to the analysis, recovery, detection, or other qualitative or quantitative determination of an analyte in a particular medium. The medium is generally fluid, typically aqueous. The term, "captured", refers to a state of being removed from a medium onto a surface.
The term "biomolecule" refers to all molecules of biological origin, be they natural or engineered. This also includes synthetic or artificial molecules that have biological-like characteristics using nanotechnology, IC based fabrication technology, molecular imprinting and the like.

Attachment groups for immobilization of the glycans on the support and in particular on the surface of a monolayer coated on the solid support comprise, for example, carboxylic-, primary amino-, alcohols, epoxy-, and isothiocyanate.

The solid support of the glycoarray of the invention can be any material on which a glycan as described herein below can be coated on. In particular, the term "solid

support" refers to any material insoluble in a medium containing a target molecule or biological molecule that is desirably captured in accordance with the invention. A preferred embodiment would be a 2 dimensional surface. Another preferred embodiment would be the use of micro and mesomaterials such as porous and non-porous, microbeads and microparticles which are used as carriers which are subsequently used in the preparation of glycoarrays. The term "target molecule" in this context refers to a molecule, present in a medium, which is the object of attempted capture. Preferably, the solid support is a support on which a self-assembling monolayer can be coated on. In that case the glycan is immobilized on the sensing surface of the self-assembling monolayer. Preferably, said solid support is silicon, glass, mica, plastic, gallium arsenide, platinum, silver, copper, gold or a combination of any one thereof, most preferably a combination of teflon and gold, the latter providing the support for binding the SAM.

In principle, the sensing surface on which the glycans are immobilized can be produced according to methods well known to the person skilled in the art such as described in WO90/05303, which also provides examples for appropriate attachment groups, spacers, solid supports and detection methods which can be employed in accordance with the present invention, if necessary in modified form. Similarly, the means and methods described in WO92/10757 can be adapted, modified and used in accordance with the present invention.

In a particular preferred embodiment of the glycoarray of the invention, said sensing surface comprises biotin and streptavidin or derivatives thereof. Such derivatives are described for example in WO91/07087 as well as methods that may be adapted in accordance with the present invention. In particular, it is preferred that streptavidin forms a nearly 100% surface coverage which prevents direct interaction of a sample with the surface below the sensing surface. Accordingly, it is preferred that X is biotin or a derivative thereof. In particular, the invention covers the design and application of arrays of glycans of type G-Y-X (1) on the surface of sensing elements with intermediate affinities (*matrixes*) of type arrays being described in patent application WO 97/49989 (PCT/EP97/03317) the disclosure content of which is specifically incorporated herewith. One embodiment of the arrays described in WO97/49989 is named XNA on Gold™ which is the registered trade mark of the

affinity array biochip developed by INTERACTIVA Biotechnologie GmbH; see http://www.interactiva.de.

Thus, while WO97/49989 provides a conceptual basis of arrays of discrete biological sensing elements it does not specifically describe glycoarrays of the present invention. In a preferred embodiment of the glycoarray of the invention, said sensing elements have a density between 5 and 60 fmol/cm^2 on the sensing surface.

As already indicated above the solid support of the glycoarray of the present invention is preferably coated with the sensing surface of a self-assembling monolayer. The term "self-assembled monolayer" refers to a relatively ordered assembly of molecules spontaneously chemisorbed on a surface, in which the molecules are oriented approximately parallel to each other. Each of the molecules includes a functional group that adheres to the surface, and a portion that interacts with neighboring molecules in the monolayer to form the relatively ordered array. See Laibinis, Science 245 (1989), 845; Bain, J. Am. Chem. Soc. 111 (1989), 7155-7164; Whitesides, J. Am. Chem. Soc. 111 (1989), 7164-7175.

The principle of self-assembling monolayers (SAMs) as well as immobilization technologies based on SAMs are for example described in Bain, J. Am. Chem. Soc. 111, (1989), 321-335; Porter, J. Am. Chem. Soc. 109, (1987), 3559-3568; and Bain, J. Am. Chem. Soc. 111, (1989) 7155-7164. Usually, the self-assembling monolayer consists of hydrocarbon chains, preferably nonbranched, optionally interrupted by hetero atoms, and of a length exceeding 10 atoms, preferably containing 16-30 atoms.

A number of different self-assembling based immobilization schemes have been developed including dendrimer based (Watanabe, et al. 1994 JACS 116: 8855-8865; Wells, et al. 1996 JACS 118: 3988-3989), electrostatic interaction based (Decher, et al., 1992 Thin solid films 211: 831-841; Cheung, et al. 1994 Thin solid films 244; 985-991), phosphonate based (Thompson 1994 Chem Mater. 6: 1168-1175), long chain alkane silane based (Netzer, et al. 1983 JACS 105: 674-676) and long chain alkane thiol based.

In a preferred embodiment the self- assembled monolayer is a self-assembled mixed monolayer which means that it is heterogenous self-assembled monolayer, i.e. one made up of a relatively ordered assembly of at least two different molecules.

The term "linker" refers to the moiety that links the surface attachment group to the glycan. This moiety serves one or more of the following functions: improving the solubility, reducing nonspecific binding of itself, reducing nonspecific binding of components in the sample, improving presentation of glycan, improving stability of the glycan or any combination thereof. In the case where a self-assembling monolayer is employed, the long chain alkane would be considered part of the linker.

Preferably, the linker group of the present invention is a monomeric, oligomeric or polymer-based spacer for presentation of glycan display, optionally containing a fragment which is cleavable by chemical and/or physical methods.

Monomeric, oligomeric and polymer-based spacers comprise polyalkyl (C1 to C18), polyethyleneglycol (tri, penta, hexa), polyaryl, polyacrylate, polyacrylamide and siloxanes.

Fragments which are cleavable by chemical and/or physical methods include but are not limit to ditnotrophenyl and other photocleavable groups, any amide, any group capable of beta-elimination reaction, esters, acid- or base hydrolysable.

The term "glycan" refers to any saccharide or oligosaccharide, in free form or attached to another molecule and is defined herein as a polyhydroxyaldehyde or a polyhydroxyketone or a larger compound that can be hydrolyzed into these units. The term glycan and carbohydrate are used interchangeably herein. Glycan also refers to any natural or non-natural non-carbohydrate compounds that mimic the physical, structural and chemical characteristics including the biological activities of natural or engineered functions of saccharides, and any combinations thereof.

In this context, an "oligosaccharide" is defined as a branched or linear chain of monosaccharides attached to one another via glycosidic linkage (Voet, Biochemie, VHC (1992)).

The term "monosaccharide" is defined herein as a carbohydrate that cannot be hydrolized into a simpler unit. A monosaccharide can be of two types containing a carbonyl at the end of the carbon chain, termed an aldolase, or at the inner carbon, termed a ketose (Voet, Biochemie, VHC (1992)). Common animal monosaccharides include for example:

- Sialic acids: a family of nine-carbon acidic sugars the most common of which is N-acetyl neuraminic acid
- Hexoses: six carbon neutral sugars, such as glucose, galactose and mannose.
- Hexosamines: Hexose with an amino group at the 2-position, which can be either free or, more commonly, N-acetylated; N-acetylglucosamine, and N-acetylgalactosamine.
- Deoxyhexoses: six-carbon neutral sugars without the hydroxyl group at the 6-position, for example, fructose.
- Pentoses: Five-carbon sugars such as xylose.
- Uronic acids: Hexose with a negatively charged carboxylate at the 6-position such as glucuronic acid and iduronic acid.

These monosaccharides dominate eukaryotic glycobiology but numerous variants can be found in lower organisms some of which are involved in numerous disease states (Schmidt, (1996) 5th edition, p. 2 Wm C. Brown, New York; Kahn, Infectious Immunity 64 (1996), 2649-2656; Mengeling, Current Opinion in Structural Biology 8 (1998), 572-577).

The term "glycan" also includes glycoconjugates. The term "glycoconjugate", abbreviated G, refers to a molecule comprised of a glycan moiety and possibly a non-glycan moiety, such that the interaction between the glycan and non-glycan moiety is sufficiently strong so as not to be released during the binding assay unless this is done by design. In this context the term "non-glycan" moiety refers to any molecule of natural and non-natural origin including: naturally occurring macromolecules such as: proteins, lipids, RNA, DNA, biopolymers and the like, non-natural macromolecules constructed using biological-like schemes such as: LNA, PNA, peptoids and the like, non-natural macromolecules with biological-like feature(s) produced using man-made engineering and fabrication design principles such as nano-injection molding or stamping, molecular imprinting or templating, as

well as sequential-reaction based nanomaterials such as dendrimers, rosettes, star-fish and the like.

Many natural bioactive molecules are glycoconjugates, and the attached glycans can have dramatic effects on the biosynthesis, stability, action and turnover of these molecules in the intact organism. Several classes of enzymes have been shown to be involved in the synthesis and degradation of glycans including: glycosidases that catalyze the hydrolysis of glycosidic bonds in a glycan, exoglycosides that catalyze the cleavage of an internal glycosidic linkage in an oligosaccharide, endoglycosidases that catalyze the cleavage of a monosaccharide from the outer (nonreducing) end of a glycan or glycoconjugate, glycotransferases that catalyze the addition of monosaccharides and proteases that catalyze the cleavage of peptide bonds thereby altering the structural conformation of the glycoconjugate exposing different saccharides. Analysis of these enzyme pathways and their glycans and glycoconjugate products have been used to delineate numerous disease states, as diagnostic tools and in the development of therapies (Kornfeld, Annu. Rev. Biochem. 54 (1985), 631-664; Van der Eijnden, Curr. Opin. Struct. Biol. 3 (1993), 711-721; Mellors, Trends in Biotechnology 12 (1994), 15-18).

Preferably, the glycan moiety is a moiety of a natural or chemically or enzymatically prepared carbohydrate molecule selected from the group consisting of monosaccharide, oligosaccharide, polysaccharide, glycolipid, glycoprotein, glycocompounds of any type with natural, modified natural or non-natural molecule that mimics the structure and/or activity of carbohydrates and any of their combinations.

In particular, carbohydrate molecules that can be used in accordance with the present invention comprise the penta saccharide GM1 for binding assay of cholera toxin, fragments of surfaces saccharides of salmonella for binding assay of anti-bodies, influenza HA antigen (NeuAc) for inhibitors development, the saccharides CM2/GD2, Sialyl-Tn for binding assay of cancer markers, and inhibitors development, Galα1,3Gal as marker for porcine xeno-transplants, Sialyl-lewis carbohydrates for binding of selectins, Glycomimentics compounds for drug development and effectivity screening, CD15s, CD21, CD34, CD45, CD57, CD58, CDw75, HSA for binding assay of lectins in the immunosystem, and Neu5Acα2-3Galβ1-4Glc for binding assay of Heliobacter pylori.

Some glycans and glycoconjugates intermediates are not yet commercially available. This is due to the fact that small amounts of these intermediates are present in cells making purification laborious, time-consuming and expensive. Furthermore, the preparation of synthetic glycans and glycoconjugates is time consuming and an expensive process due to the inherent difficulties presented by this class of multifunctional compounds. This is due to the fact that stepwise synthesis of discrete carbohydrate analogues involves many protection and deprotection steps and that glycosylation creates a new stereocenter at the anomeric carbon (which can either be alpha or beta) and so far the available methods typically have poor yields and long reaction times as compared with the coupling reactions of peptide bond and phosphodiester formation (Khan, Mol. Glycobiology (1994), 206-223). Alternatively, enzymatic synthesis of oligosaccharides has shown promise in overcoming these drawbacks by eliminating the need of protecting groups and the "built-in" stereo specificity. However, this scheme also has limitations such as the limited number of enzymes available, enzyme stability and specificity variation, the limited quantity and high cost of the enzymes (Palic, Trends in Glycoscience and Glycotechnology 8 (1996), 37-49; Wong, Angew. Chem. Intl. Ed. Engl. 34 (1995), 412-432). Approaches that reduce the amount of material needed to assay any given glycan or glycoconjugate and that do not require target purification are sorely needed.

Thus, in a preferred embodiment of the glycoarrays of the invention said glycans are modified by use of chemical, physical and/or enzymatic techniques to allow the modification of glycans immobilized on glycoarrays including but not limited to the sequential synthesis and/or degradation of said immobilized glycans. The advantages of combining array and modification technologies are as follows: (i) reduces reagent usage, (ii) small size provides better control over modification reaction conditions, (iii) a wide range of modification conditions can be tested simultaneously, (iv) improved control over experimental conditions, (v) increased throughput, (vi) on-chip standards provides a method for analyzing experimental variation which reduces experimental error, and (vii) probe enzyme specificity and incorporation efficiency.

In a particular preferred embodiment of the present invention the array is an array of discrete sensing elements of carbohydrate molecules (glycoarray) wherein carbohydrate derived molecules of the type G-Y-X (1) are immobilized on the sensing surface of a self-assembling monolayer coated on a solid support (glycochip) and wherein

X is an attachment group for immobilization on the surface of the monolayer;

Y is a monomeric, oligomeric or polymer-based spacer for presentation of carbohydrate display, optionally containing a fragment which is cleavable by chemical and/or physical methods;

G is a moiety of a natural or chemically or enzymatically prepared carbohydrate molecule selected from the group consisting of monosaccharide, oligosaccharide, polysaccharide, glycolipid, glycoprotein, glycocompounds of any type with natural, modified natural or non-natural structure mimetics of carbohydrates and any of their combinations.

The arrays of carbohydrate molecules are (should be named) glycoarrays and the platform (*matrixes*) which bear glycoarrays are (should be named) glycochips.

The invention described here applies microarray analysis to the next level of complexity namely carbohydrates (or glycans). The term "glycome" refers to all glycans expressed in a cell and represents the next level of increasing biological complexity. This is due to the fact that the chemical structure of the monosaccharide bond allows two possible linkages and the formation of branched structures, as apposed to nucleotides and proteins which form only linear polymers and have only one basic type of linkage. As a result, the structural complexity of glycans is several orders of magnitude greater than proteins and DNA (Lis, European Journal of Biochemistry 218 (1993), 1-27; Laine, Glycobiology 4 (1994), 1017-1023).

In addition to this chemical complexity, a common feature of protein glycosylation is that at any given glycosylation site on a given protein, synthesized by a particular cell type, a range of variations can be found in the structure of the glycan. These minor glycosylation variants are referred to as microheterogeneities. Thus, a given glycoprotein can exist in numerous glycoforms, i.e. different molecular forms of a

glycoprotein resulting from variable glycan structure and/or glycan attachment site occupancy. The origin of this microheterogeneity lies in the dynamics of the sequential nature of the glycosylation process. Microheterogeneity is typically analyzed using traditional gel electrophoresis and comparing the number of diffuseness of the bands. The method is time consuming and reproducibility is often poor (Jackson, A Laboratory guide to glycoconjugate analysis. Biomethods, 9 (1997), Birkhauser, Basel, Switzerland; Lennarz, Methods in Enzymology 230 (1994), Academic Press, New York). The presence of an appropriate band on a gel indicates that the biomolecule is intact but does not probe the biological activity (see mRNA and protein expression discussion above).

The glycoarrays of the present invention make it possible to investigate the interaction of all possible types of glycans with their natural or non-natural ligands. In particular, one of the aims of the present invention is to mimic natural "sugar" coating that exists on biological surfaces in order to allow the analysis of ever more intricate biological interactions.

In a preferred embodiment the glycoarray is designed so as to have variable glycan densities, i.e. sub-saturating densities of glycan. Glycan densities could be varied from slightly 0 above to 100% or near 100%. Furthermore, the local density on the micron scale could be modulated by immobilizing subsaturating amounts of nanomaterials, i.e. dendrimers and the like, of known size and with known, variable glycan densities to provide locally high surface densities while maintaining a low surface concentration. Moreover, glycan gradients could be created over the glycoarray, each discrete spot having a constant surface concentration to provide a strategy for screening density dependency. Alternatively, gradients could be made over each individual discrete region. The surface density could be further controlled by synthesizing multiple repeats of the same oligosaccharide or any combination thereof.

In another preferred embodiment the glycoarray contains two or more glycans coimmobilized on the support. Alternatively, this can be done by immobilizing multimers composed of oligosaccharide units of variable unit composition and linked

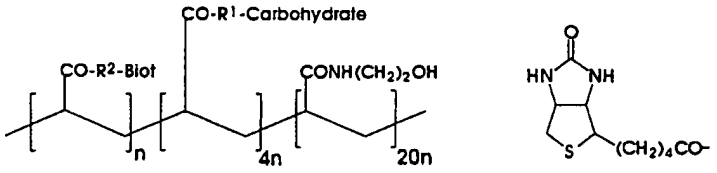
together as linear chains, as branched chains, with natural biopolymers, with synthetic polymers, as dendrimers, rosettes, star-fish or any combination thereof.

Up until about a decade ago only a few human lectins had been identified. The term "lectin" refers to a protein that specifically recognizes and binds to glycans without catalyzing a modification of the glycan. However, during the past decade the number has rapidly increased to several hundred members and have been implicated in a variety of processes including cellular differentiation during development (Leffler 1997, Trends in Glycosci Glycotechnol. 9: 31-40; Lobsanov, et al., 1997 Trends in Glycosci Glycotechnol. 9: 145-154), a wide variety of physiological responses, regulating leukocyte trafficking and adhesion (Springer 1995 Annu. Rev. Physiol. 57: 827-872), cell-cell and cell-matrix interactions (Drickamer, et al., 1993 Annu. Rev of Cell Biol. 9: 237-264) and immune regulation (Dohring et al., 1997 Crti. Rev Immunol. 17: 285-299; Weis, et al., 1998 Immunol. Rev. 163: 19-34). The vast majority of these interactions have as yet to be characterized. It is known that lectins are expressed tissue specifically in sets and that lectins are not evenly distributed (Horstkorte, et al. 1993 J. Cell Biol. 121: 1409-1421).

Despite the fact that numerous human proteins with lectin-like activity have been identified few have been assigned biological functions. Most of the evidence for the involvement of these molecules comes from animal model studies or human hereditary diseases. These studies unfortunately provide only hints as to the possible mechanism of action, while the model organism are to simple to be of any use. The glycoarrays described herein could be useful in bridging this gap.

Preparation of glycoarrays or glycochips includes the attachment of optimally designed molecules of type (**1**) onto the surface of the solid support, preferably onto the surface of the sensing elements, via interaction of group **X** in (**1**) and of **X**-recognizing molecules being covalently linked to the surface of the solid support or sensing elements. Methods for the preparation of chemical derivatives and analogues of glycans are well known to those skilled in the art and are described in, for example, Beilstein, Handbook of Organic Chemistry, Springer edition New York Inc., 175 Fifth Avenue, New York, N.Y. 10010 U.S.A. and Organic Synthesis, Wiley, New York, USA. As typical examples the protocol for the loading of monodentate

biotinylated carbohydrate conjugates of type (**2**) and of polydentate biotinylated polyacrylamide (PAA) based carbohydrate conjugates of type Carbohydrate-PAA-Biotin (**3**) is presented in the experimental part.



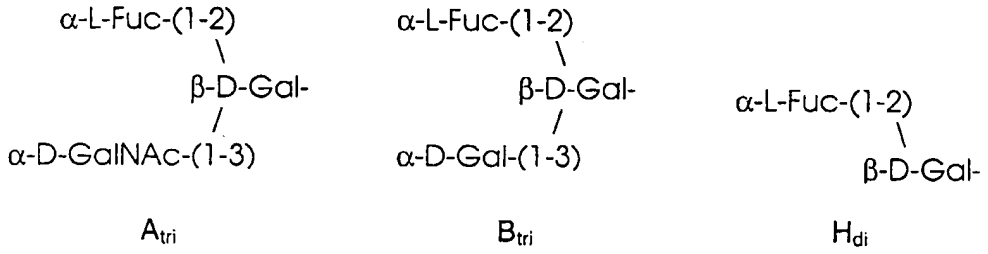
(**2**) (**3**, Carbohydrate-PAA-Biotin) **Biot**

R^1, R^2 and R^3 are spacer-arms

Loading of glycoconjugates onto chip surface proceeds due to direct interaction of the attachment group **X** and **X**-recognizing molecules being covalently linked in known value to the surface of sensing elements. Nevertheless the amount of glycan ligands on the surface of glycochip can be characterized by using of respective labeled antibodies (see figures 1-4 and discussion below) and lectins or other glycan binding proteins, as well as by using a number of nonlabel detection principles including but not limited to quartz crystal microbalances, optical techniques such as optoaucostics, reflectometry, surface plasmon resonance (SPR). If it is possible to disrupt the binding interaction by chemical or physical methods the group **Y** or to destroy the binding between group **X** and **X**-recognizing molecule on the surface of sensing element, the detection of carbohydrate molecules can be performed by using of mass-spectrometry.

The detection limit of carbohydrate ligands on the surface of glycochips might be very small (*low*) and depends on the used visualization technology. Particularly, Figures 1 and 2 show the revealing of A_{tri}-ligands on the surface of glycochip sensibilized with a series of glycoconjugates of type (**3**) by using of the combination of mouse monoclonal A9 antibodies (anti-A_{tri}) with anti-mouse-Cy5 conjugate (Fig. 1) or anti-mouse-alkaline phosphatase (AP) conjugate (Fig. 2). The second protocol permits reliable detection of much smaller amounts of glycan ligand.

Revealing of glycan ligands under these protocols is not accompanied by non-specific events. For example the Figures 1 and 2 also show the absence of tracing with A9 antibodies (anti-A_{tri}) for the chip spots containing B_{tri}-PAA-Biotin conjugate of type (**3**) which in turn can be revealed with B16 antibodies (anti-B_{tri}) as it is shown on the Fig. 3.



A_{tri} B_{tri} H_{di}

Sensitivity of glycan assaying by using of glycochips depends also on the structural characteristics of glycan of type (**1**) employed in the design of glycoarrays. Structure of glycans determine the spatial presentation of glycan ligand which is important for better recognition. Fig. 4 shows the revealing of A_{tri}-ligands in the spots of glycochip sensibilized with different amounts of monodentate A_{tri}-conjugate of type (**2**) [R^3 = -$(CH_2)_3NHCO(CH_2)_5NHCO(CH_2)_5NH$-] by using of the combination of mouse monoclonal A9 antibodies (anti-A_{tri}) and anti-mouse-AP conjugate. In case of the conjugates of same type but with shorter spacer R^3 [for example -$(CH_2)_3NHCO(CH_2)_5NH$- or -$(CH_2)_3NH$-] no tracing was observed.

Having described an example for the preparation of a glycoarray and glycochip of the invention it is clear that the present invention also relates to a method of producing the above described glycoarrays and glycochips comprising immobilization of a glycan derived molecule as defined above on a solid support and preferably on a sensing surface. This can be achieved by either arraying premade, preferably purified, glycans onto the support, by assembling glycans directly on the support, or by modifying, preferably enzymatically premade glycans before immobilization or by modifying, preferably enzymatically, already immobilized glycans. Preferably the sensing surface is coated with streptavidin or with a derivative thereof and the immobilization of the glycan is achieved via biotin or a

derivative thereof. For example, the sensing surface of XNA on Gold™ can be used except that XNA is replaced by the carbohydrate derived molecule.

The glycoarrays according to the present invention can be used to probe the biological activity of biomolecules. This is performed by analyzing the binding pattern of a given biomolecule on the glycoarray which reveals the saccharide binding specificity, thus probing the functionality of the biomolecule. This information is compared with the expected binding pattern if this is known. Alternatively, the entire glycoarray binding pattern or "fingerprint" can be compared using normal or wild type standard glycoarray "fingerprint" of the same molecule. This comparative approach has been shown to be very useful when studying complex multiparameter systems (Todd, et al., 1998 Tibtech 16: 250-258; Lundstrom, et al., 1991 Nature 352: 47-50).

Glycoanalysis is a laborious process involving a combination of chemical and enzymatic cleavages followed by mass spectrometric analysis (Bierman, et al. 1989 Analysis of carbohydrates by GLC and MS; Hounsell, ed. 1993. Glycoprotein analysis in biomedicine. Methods in Mol. Biol., vol. 14 Humana Press, Totowa, New Jersey). A wide range of techniques are used to analyze and sequence glycans including derivatization, metabolic labeling, use of endo- and exoglycosidases, composition analyses, various forms of glycan release and chromatographic separation, chemical methods for linkage position analysis, and ultimately to mass spectrometry and NMR spectroscopic methods for complete sequence determination. This process is typically repeated several times with minor variations before the sequence is delineated, this despite a large body of empirical experience. The inability to prepare standards on-demand due to the difficulties in synthesizing oligosaccharides exaggerates the problem. Despite these drawbacks, it has been possible to delineate a large number of saccharide sequences and synthesize them, which serves as the basis for glycoarray technology.

Glycosylation and glycobiology have been studied in a number of model organisms including yeast, dictostelium, C. elegans, sea urchins, drosophila and xenopus (Kukuruzinska, et al., 1987 Annu. Rev. Biochem 56: 915-944; Kuwabara, 1997 Trends in Genetics 13: 455-460; Alves, et al., 1998 Glycobiology 8: 939-946). Each system has advantages and disadvantages. However all these simple organisms

suffer from one major drawback, they do not display the complex genetics, development and behavior critical for understanding the role glycans play in higher organisms. As an alternative, researchers have constructed a variety of cell lines and transgenic mice including expressing glycosidases, masking glycosyltransferases, competing glycosyltransferases, and overexpressing endogenous glycosyltransferases (Gridley, et al., 1987 Trends in Genetics 3: 162-166; Asano, et al., 1997 EMBO J. 16: 1850-1857; Hennet, et al., 1998 PNAS 95: 4504-4509). These studies indicate that glycans fulfil important physiological functions, however the systems are too complicated to delineate the mechanisms involved. The difficulty lies in the high degree of redundancy that glycans seem to exhibit which requires large numbers of experiments to make the results statistically meaningful.

One advantage of the glycoarray according to the present invention is the ability to assay the same interaction in multiplicity under essentially identical conditions, making statistical analysis of the natural variation possible. Furthermore, analytically identical glycan samples as well as natural microheterogeneous glycoconjugates could be arrayed to test and define glycan variation in biological relevant terms, i.e. correlated to a binding activity.

Furthermore, as explained above the present invention relates to a method for discriminating complex biological samples using a glycoarray or glycochip of the present invention preferably in which constituents bound to the glycoarray are determined by label or non-label detection systems.

The term "determining" in this context refers to quantitative or qualitative analysis of a species via, for example, spectroscopy, ellipsometry, piezoelectric measurement, immunoassay, and the like.

Detection can be achieved using any one of a number of techniques well known to the person skilled in the art including time resolved fluorescence, fluorescence, radiolabel, chemiluminescence, interferometry, luminescence, or FRET based systems (Kessler ed. 1992 Nonradioactive labeling and detection of biomolecules Spring Verlag, Berlin).

Preferably, a label detection system employs fluorescence, chemiluminescence, bioluminescence or epifluorescence or a non-label detection system comprises measuring the increase in mass or thickness on the surface of the glycoarray.

In a preferred embodiment of the present invention said surface mass increase detection techniques are selected from but not limited to the group consisting of quartz crystal microbalances, optoaucostics, reflectometry, ellipsometry, SAW, surface plasmon resonance, mass spectrometry, capacitive and amperometric techniques.

In a particularly preferred embodiment the glycan or glycoconjugate is directly immobilized to the solid support, the target binding is detected using any one of a number of fluorescence based assay techniques well known to the person skilled in the art.

In a further preferred embodiment of the method of the invention said detection is performed in imaging mode with a CCD camera or a scanning based device. Scanning probe microscopy immunoassay is described for example in WO92/15709.

The described glycoarrays and glycochips are useful and indispensable tools for assaying of glycan recognition of different types including that ones which can be applied in the development of a variety of diagnostics of health states. Glycan recognizing molecules include: labeled and non-labeled antibodies, selectins, bacterial and viral adhesins and other lectins, carbohydrate processing enzymes (f.ex. glycosyl-, sulfo-, acyl-transferases, glycosidases, etc.), carbohydrates which participate in carbohydrate-glycan interactions and the like. The involvement of oligosaccharides in selectin-mediated cell-cell recognition by the immune system in response to inflammation (Lasky, Science 258 (1992), 964-969), and sperm-cell recognition during fertilization (Miller, Nature 357 (1992), 589-593) are but a few examples. It is also known that modifying the expression of glycosides and glycosyltransferases interferes with normal development. Glycochips can be used also in the experiments with different cells and their fragments including, but not limited to, microorganisms, bacteria and viruses containing the listed above glycan recognizing molecules and any other glycan binding sites.

The described glycoarrays and glycochips can be used for development of a variety of diagnostics of health states. These could be assays to reveal and evaluate the amounts of carbohydrate recognizing molecules and cells of listed above types including, but not limiting to, immunoassays, serology assays, diagnostics of infectious diseases and the diseases caused by defects and other abnormalities of carbohydrate processing enzymes.

Such assays preferably are based on biological binding between the glycan immobilized on the support and a molecule to be captured. In this context the term "biological binding" refers to the interaction between a corresponding pair of molecules, i.e. binding partners, that exhibit mutual affinity or binding capacity, typically specific or non-specific binding or interaction, including biochemical, physiological, and/or pharmaceutical interactions. Biological binding defines a type of interaction, preferably by recognition regions, that occurs between pairs of molecules including proteins, nucleic acids, glycoproteins, carbohydrates, hormones and the like. Specific examples include antibody/antigen, antibody/hapten, enzyme/substrate, enzyme/inhibitor, enzyme/cofactor, binding protein/substrate, carrier protein/substrate, lectin/carbohydrate, receptor/hormone, receptor/effector, complementary strands of nucleic acid, protein/nucleic acid repressor/inducer, ligand/cell surface receptor, virus/ligand, etc.

The term "non-specific binding" refers to interaction between any species, present in a medium from which a target or biological molecule is desirably captured, and a binding partner or other species immobilized at a surface, other than desired biological binding between the biological molecule and the binding partner.

The term "binding partner" refers to a molecule that can undergo biological binding with a particular biological molecule. For example, Protein A is a binding partner of the biological molecule IgG, and vice versa.

The term "recognition region" refers to an area of a binding partner that recognizes a corresponding biological molecule and that facilitates biological binding with the molecule, and also refers to the corresponding region on the biological molecule.

Recognition regions are typified by sequences of amino acids, molecular domains that promote van der Waals interactions, areas of corresponding molecules that interact physically as a molecular "lock and key", and the like.

Glycans and glycoconjugates have been shown to have numerous biological activities e.g. cell adhesion, cell-cell interactions, pathogen-host interactions, toxins in cancer and inflammation processes, for example, carbohydrate binding proteins such as selectins are believed to play a critical role in immune responses including inflammation (Springer, Nature 349 (1991), 196-197; Philips, Science 250 (1990), 1130-32) and as a modifier of the activity, stability and biological activity of proteins, and as immunogenic substances which have potential for vaccination against different diseases. An extensive literature has been developed during the last few years in this field (Montreuil, Glycoproteins and Disease (1996), Gabius, Glycoscience (1997)). Specific carbohydrate ligands have been identified and have been used to control inflammation, immunosuppression, etc. through their interaction with selectin proteins and/or other lectins (US-A-5,576,305; US-A-5,374,655). Other glycoproteins have also been shown to be useful in suppressing mammalian immune responses (US-A-5,453,272). As the example the protocol for assaying the α-galactosidase by using the glycochip sensibilized with B_{tri}-ligand containing conjugates of type (**3**) is given in the Experimental. Treatment with α-galactosidase cause the transformation of B_{tri}- into H_{di}- ligands that is visualized under the using of the combination of mouse monoclonal B16 antibodies (anti-B_{tri}) and anti-mouse-AP conjugate (Fig. 5).

Numerous human disease states are known to involve acquired (noninherited) changes in glycosylation and/or in the recognition of glycans, i.e. human glycophathologies. These alterations have been used as the basis for developing prodiagnostics, diagnostics, as therapeutics and/or as lead compounds for therapeutic drug design. These include cardiovascular disorders (Lowe, J. Clin. Invest. 99 (1997), 822-826), atherosclerosis (Bartlett, Atherosclerosis 138 (1998), 237-245), inflammatory skin diseases (Austrup, Nature 385 (1997), 81-83; Fuhlbrigge, Nature 389 (1997), 978-981), diabetes mellitus (Vlassara, Diabetes 45 (1996), 65-66; King, Endocrinology Metabolism Clinics North American 25 (1996), 255-270), gastrointestinal infections (Karlsson, Curr. Opin. Struct. Biol. 5 (1995), 622-635; Zopf, Lancet 347 (1996), 1017-1021; Thompson, Glycoconjugate 14 (1997), 837-845; Ilver, Science 279 (1998), 373-377), ulcerative colitis (Reid, Histochemical Journal 16 (1984), 235-251), hepatomas and liver disorders

(Maekawa, J. Clin. Invest. 990 (1992), 67-76), cold agglutinin autoimmune disease (Kewitz, Glycoconjugate Journal 12 (1995), 714-720), rheumatoid arthritis (Parekh, Nature 316 (1985), 452-457; Korbata, Glycobiology 1 (1991), 5-8), nephrotic syndromes (Charest, PNAS 82 (1985), 8505-8512), neurological diseases (Fredman, Neurochemistry Research 22 (1997), 1071-1083; Jacobs, J. Infect. Dis. 175 (1997), 729-733; Carpo, J. Neuro. Sci. 155 (1998), 186-191), Alzheimer's disease (Goedert, Nature 383 (1997), 550-553), bronchial asthma (Hitata, Am. J. Resp. Cell Mol. Biol. 18 (1998), 12-20), acute respiratory distress, shock, trauma and sepsis (Lowe, J. Clin. Invest. 99 (1997), 822-826) and cystis fibrosis (Mawhinney, Carbohydrate Research 235 (1992), 179-197; Imundo, PNAS 92 (1995), 3019-3023).

A variety of inherited diseases in glycan biosynthesis have been identified including: carbohydrate-deficient glycoprotein syndromes (Jaeken, J. Inherited Diseases 16 (1993), 813-820; Freeze, J. Pediat. 133 (1998), 593-600), leukocyte adhesion deficiency syndrome II (Tan, Am. J. Hum. Gen. 59 (1996), 810-817), congenital dyserythropoietic anemia type II (Chui, Cell 90 (1997), 157-167), galactosemia (Stibler, Acta Paediactrics 86 (1997), 1377-1378), abnormalities in proteoglycan synthesis (Quentin, PNAS 87 (1990), 1342-1346), deficiencies in blood group gylcosyltransferases (Lowe, The molecular basis of blood diseases, ed. Stamatoyannopulos et al., W.B. Sanders, Orlando, Florida (1999)), defects in glycan degradation (Jourdian, New Compr. Biochem. 30 (1996), 3-54) and lysosomal disorders (Platt, Science 276 (1997), 428-431).

Thus, the present invention also relates to a method of diagnosing a disease comprising contacting a sample from a subject with the glycoarray or glycochip of the invention. Preferably, the method comprises any one of the afore-described methods for discriminating complex biological samples, in particular the described label or non-label detection systems. In principle, any sample can be analyzed in accordance with the methods of the present invention. Naturally, when diagnosing a disease the sample is preferably derived from a human or animal, tissue or bodily fluid, selected from the group consisting of blood, serum, urine, milk, sweat, exhaled air, skin, bone marrow, cerebrospinal fluid, synovial fluid, amniotic fluid and

lymphatic fluid. The disease that may be analyzed in accordance with the method of the present invention can be any disease which is related to the presence or absence of carbohydrate binding molecules, normally proteins or processing enzymes and comprise diseases selected from the group consisting of genetic disorders, autoimmune diseases, arthritis, infectious diseases, cancer, heart disease, drug abuse HIV, BSE and lung disease.

In accordance with the methods of the present invention, the samples to be analyzed can be discriminated by correlating the values from the entire array using for example pattern recognition and compared to a reference sample. This increases the speed and reduces the time required to perform assays, thereby reducing costs, all of which are objects of this invention.

In one embodiment of the invention, the glycoarrays and glycochips are used in combination with neural network analysis as a diagnostic tool to discriminate complex samples, such as serum samples. The ubiquitous presence of glycan binding molecules in all living organisms provides a nearly universal means for identification of complex biological samples. The complex biosynthetic pathways used to synthesize these glycan binding molecules are effected by subtle changes in their environment. These changes lead to a series of complex global modifications in the composition and thereby the structure of the glycan binding molecules.

Furthermore, altered glycosylation pattern is a feature common to all types of cancer cells and a number of glycan structures are well-studied markers for tumor progression (Feizi, Nature 314 (1985), 54-57; Dennis, CRC Press (1992), 161-194). Many of the original "tumor-specific" antibodies were directed against carbohydrate epitopes. Glycosylation can be altered in many ways including altered branching of N-glycans (Fukuda, Cancer Research 56 (1996), 2237-2244; Taniguchi, Glycobiology 6 (1996), 691-694), changes in the amount, linkage and acetylation of sialic acid residues (Dennis, Nature 300 (1982), 274-276; Yamashita, Cancer Research 55 (1995), 1675-1679), altered glycosaminoglycans (Lesley, Glycoconjugate 14 (1997), 611-622), modified mucin glycosilation (Bhavanandan, Glycobiology 1 (1991), 493-503; Taylor-Papadimitriou, Immunology Today 18

(1997), 105-107), altered Lewis structures (Nakamori, Dis. Colon Rectum 40 (1997), 420-431), modified expression and shedding of glycosphingolipids (Hakomori, Cancer Research 56 (1996), 5309-5318), increased galectins (Cornil, J. Cell Biol. 111 (1990), 773-781) and altered expression of blood-group related structures (Lee, New England J. of Medicine 324 (1991), 1084-1090).
Neoplasia involves alterations in gene expression that dramatically alter the growth, invasion and metastasizing capabilities of cells. The highly selective glycosylation changes seen in tumor cells that survive the selection process indicates that glycosylation plays a crucial role in these processes. Understanding these processes is of great medical importance since invasion and metastasis is the most common cause of death in cancer patients. Unfortunately, cellular heterogeneity in tumors and inherent genetic instability make it difficult to determine the functional consequences of specific glycosylation changes in such complex systems.

The presently large number of terminal glycan chain modifications continues to expand the structural diversity of this class of biomolecules. The overlapping biosynthetic pathways, combined with the multiplicity of organs and tissues where these structures are found, imply that a correspondingly large number of experiments will be needed to delineate glycan function. Current assay technology has been unable to fulfil these demands.
Alteration in glycosylation patterns have also been shown during development and in numerous physiological responses including: fertilization (Youakim, J. Cell Biol. 126 (1994), 1573-1583; Fenderson, Bioessays 12 (1990), 173-179), neurogenesis (Blum, PNAS 84 (1987), 8716-8720; Britis, Science 255 (1992), 733-736; Kiss, Curr. Opin. Neurobiol. 7 (1997), 640-646), organogenesis (Kitagawa, J. Biol. Chem. 269 (1994), 17872-17878), hematopiesis and the immune system development (Baum, J. Biol. Chem. 271 (1996), 10793-10799).
The results from these and other studies show that glycans have a modulatory role in cell-cell interactions, cell-migration patterns during development and receptor activation responses.
This invention takes advantage of this diversity in order to increase the amount of information that can be obtained, for example instead of quantitating the exact amount of a particular compound that has bound to a specific glycan molecule as

may be routinely done in conventional diagnostics. An additional object of the invention is the ability of the assay to take advantage of as yet unidentified recognition capabilities present on biomolecules for glycan molecules. These unidentified recognition elements will provide information that allow the discrimination of samples with unprecedented accuracy and presently not possible with any other diagnostic assay strategy. This complex interplay provides a wealth of data which, due to the rapid development in computer technology and signal processing techniques, can be rapidly analyzed. Moreover, the ability of glycoarrays will grow dramatically as more biomolecules are tested in the assay and their unknown recognition functions become evident.

Accordingly, the present invention also relates to a method of diagnosing the general state of health comprising any one of the above-described methods of the invention, preferably wherein a signal pattern is generated which is diagnostic of a particular state of health, said sample is a patient sample and said standard is the pattern present in a representative part of the population. The general state of health can be any relevant physiological condition which is a main amenable to diagnosis. For example, said general state of health can be selected from common mild ailments and/or health conditions with diffuse symptoms, consisting of high blood pressure, pregnancy, common colds, injuries, inflammatory reactions, mild immune suppression, doping, altitude sickness, space sickness chronic fatigue syndrome, and effects of low level toxic chemical or radiation exposure, menstrual cycles and subclinical infections.

Numerous pathogens use carbohydrate-lectin interactions in order to gain entry into their hosts. For example, bacteria and intestinal parasites, such as amoeba, mediate the sugar specific adherence of the organisms to epithelial cells and thus facilitate infection. (Liener, I.E., Sharon, N., Goldstein, I.J. eds (1986) The Lectins: Properties, functions and applications in biology and medicine. New York: Academic.). Numerous parasites and other infectious diseases synthesize glycans binding proteins for attachment and invasion of host cells including: helminths, ascaris, hookworms, malaria, amoeba, intestinal and genital flaggellates (Zaman, Handbook of medical parasitology, 2nd edition (1989); Schmidt, Foundations of

parasitology, 5th edition (1996); Mengeling, Curr. Opin. Struct. Biol. 8 (1998), 572-577). Viruses such as influenza virus (myxovirus) and Sendia virus (paramyxovirus) use a haemagglutonin protein that binds sialic acid containing receptors on the surface of target cells to initiate the virus-cell interaction (Paulsson, J.C. Interaction of animal viruses with cell surface receptors. in: The Receptors (Vol. 2) (ed. P.M. Conn), Academic Press, New York, pp. 131-219, 1985). Furthermore, the differential expression of glycan binding molecules in organisms, for example on their cell surfaces are correlated with a pathogenicity and/or host specificity. With the glycoarrays and glycochips of the present invention it is now possible to identify such organisms, for example pathogenes described above. Accordingly, the present invention also relates to a method of identifying an organism, said method comprising any one of the above described methods, wherein a signal pattern is generated that is unique to a particular organism, said sample is a biological sample from a particular organism and said standard is the pattern normally found in that organism.

Bacteria produce a variety of glycoconjugates and glycans many of which are not present in eukaryotic organisms. The best known gram-negative bacterial glycolipid is Lipid A. Exposure to Lipid A results in fever and septic shock, and can result in death. The structure is known (Raetz, 1993 J. Bacteriology 175: 5745-5753). E. coli possess the O-antigen of which 170 variants have been identified (Roberts 1996 Annual review of microbiology 50:285-315). Chlamydia trachomatis, the most common cause of blindness in the world today, makes a high-mannose-type N-glycan that is unique (Moens et al., 1997 Arch. Microbiol. 168: 169-175). Mass screening of organisms to identify unique glycan structures is not possible due to the lack of simple, inexpensive assays, despite the fact that adequate diagnostic tests are not available for many of these bacteria.

Other pathogenic microorganisms exploit host cell-surface glycoconjugates as receptors for cell attachment, tissue colonization and invasion (Karlsson 1989 Annu. Rev. Biochem 58: 309-350). These microbial adhesins present on the surface of bacteria, virus and parasites are involved in attachment to epithelial cells in the respiratory tract or the gastrointestinal tract and are required for infection (Zhang, et al., 1992 69: 861-869; Stahl, et al., 1998 Curr. Opin. Immunol. 10: 50-55).

Delineating the targets would provide useful information for understanding the biology and developing therapeutic strategies (Von Itzstein, et al. Curr. Med. Chem 4 (1996), 185-210).

Thus, the present invention also relates to the use of the glycoarrays for prodiagnostic and diagnostic screening, as well as for detection of biological warfare agents and to their use for screening for binders and to test these as antimicrobial agents.

The majority of the studies cited above pertain to human. Study of other mammalians indicate that the majority of glycan alterations exist but often show species specific glycan alterations. Thus, a preferred embodiment of the glycoarrays would be for use in veterinary science for use in areas such as identification of animal species, health testing, pro-diagnostic and diagnostic disease, use as therapeutic agents, and the like.

Furthermore, the glycoarray of the present invention can be used in the field of plant biology. In plants glycans are used extensively for structural integrity, i.e. the cell wall, as well for the purposes described above (Showalter, Plant Cell 5 (1993), 9-23). The cell wall is a source of small glycans that are used to signal patterning and morphogenesis, i.e. mammalian equivalent to hormones, as well as to signal pathogen invasion which activates a signaling cascade that turns on defense genes. Defensive oligosaccharides have between 4 and 20 saccharide residues (Hahn, Ann. Rev. Phytopath. 34 (1996), 387-412). Glycans also serve an important role in the symbiotic relationship between nitrogen-fixing bacteria and plants (Lerouge, Nature 344 (1990), 781-784; Rohrig, Science 269 (1995), 841-843). Complex plant glycans have been shown to be highly immunogenic in mammals, with the potential for inducing allergic responses (Fitchette-Laine, Plant Journal 12 (1997), 1411-1417).

Thus, an additional preferred embodiment of the invention in the field of plant science is the characterization of potentially allergenic compounds, analysis of plant signaling compounds as tools for studying plant biology and development, as pro-diagnostic state-of-health markers, for diagnosing plant infections and for analysis of

glycoconjugates produced in plants including natural compounds and those introduced by natural plant breeding or of genetically engineered plants.

The use of the glycoarrays, glycochips and methods of the present invention can be extended to a wide range of applications that require complex sample discrimination including but not limited to identification of diseases, identification of changes caused by the disease itself in the host (including but not limited to human, animal, plants and microorganisms). Complex samples containing biological material and/or degradation products including but not limited to such as food stuffs like beverages, dry foods, and the like (including but not limited to quality control, for detection of unwanted microbial growth, freshness, physical damage), as well as the control of environmental samples for microbial flora (including but not limited to microbial content and composition), pollutants and their breakdown products in air, soil and water samples. This strategy and assays based on it could also be used for monitoring fermentation processes, including but not limited to yogurt, beer, wine and the like, broths, as well as in fermentation processes in which products are produced such as biological compounds produced by microbial processes, such as insulin from genetically engineered bacteria and the like, as well as condiments made for seasoning and the like, as well fermentation processes used in the production of animal food stuffs.

A further valuable field of application for glycochips is drug discovery where they can be used in test systems for high throughput screening (HTPS) of inhibitors of glycan mediated processes and selected glycan recognizing cells and their fragments and for many other purposes.
Competitive advantage of the glycoarrays and glycochips based assays, as compared with 96-, 384- and 1536-well plates based ELISA and related protocols, are better sensitivity, specificity (reducing background) and reproducibility, use of much smaller amounts of reagents (higher level of miniaturization), possibility of the investigation of unlimited series of glycan ligands within one assay.

In a further embodiment, the present invention relates to a kit comprising a glycoarray or glycochip of the invention, and optionally suitable means for the

detection such a those described above. Thus, the kit of the invention may contain any one of the above-described glycoarrays and glycochips and further ingredients such as buffers, means for performing fluorescent or chemiluminiscent assays and other components for performing an appropriate assay. The kit of the present invention may advantageously be used for carrying out any one of the above-described methods and could be employed in a variety of applications, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in foley, etc. or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art.

Furthermore, the present invention relates to the use of any one of the above-described glycoarrays and glycochips for a diagnostic assay or preferably a diagnostic assay as described with respect to a method of the present invention.

These and other embodiments are disclosed and encompassed by the description and Examples of the present invention. Further literature concerning any one of the antibodies, methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries and databases, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

This disclosure may best be understood in conjunction with the accompanying drawings, incorporated herein by references, which show:

Figure 1: Revealing of A_{tri}-ligands on the surface of glycochip sensibilized with a series of glycoconjugates of type (**3**) at different concentrations by using of combination of mouse monoclonal A9 antibodies (anti-A_{tri}) and anti-mouse-Cy5 conjugate. Absence of non-specific tracing is shown on the example of the spots sensibilized with B_{tri}-PAA-Biotin conjugate.

*Concentration of glycan ligand in solution used for sensibilization of the chip.

Figure 2: Revealing of A_{tri}-ligands on the surface of glycochip covered with a series of glycoconjugates of type (**3**) at different concentrations by using of the combination of mouse monoclonal A9 antibodies (anti-A_{tri}) and anti-mouse-AP conjugate. Absence of non-specific tracing is shown on the example of the spots sensibilized with B_{tri}-PAA-Biotin conjugate.

* Concentration of glycan ligand in the solution used for sensibilization of the chip.

Figure 3: Revealing of B_{tri}-ligands on the surface of glycochip covered with a series of glycoconjugates of type (**3**) at different concentrations by using of the combination of mouse monoclonal B16 antibodies (anti-B_{tri}) and anti-mouse-AP conjugate. Absence of non-specific tracing is shown on the example of the spots sensibilized with A_{tri}-PAA-Biotin conjugate.

* Concentration of glycan ligand in the solution used for sensibilization of the chip.

Figure 4: Revealing of A_{tri}-ligand on the glycochip sensibilized with monodentate A_{tri}-conjugate of type (**2**) [R^3 = $-(CH_2)_3NHCO(CH_2)_5NHCO(CH_2)_5NH-$] at different concentrations by using of the combination of mouse monoclonal A9 antibodies (anti-A_{tri}) and anti-mouse-AP conjugate.

* Concentration of carbohydrate ligand in the solution used for sensibilization of the chip.

Figure 5: Revealing of B_{tri}-ligands on the surface of treated and non-treated with α-galactosidase glycochip sensibilized with B_{tri}-PAA-Biotin conjugates (type **3**) by using of the combination of mouse monoclonal B16 antibodies (anti-B_{tri}) and anti-mouse-AP conjugate.

The examples illustrate the invention.

EXPERIMENTAL

Materials. Phosphate buffer tablets, Tween, BSA were from Sigma (USA). Glycoconjugates of types (**2**) and (**3**) were the products of Syntesome GmbH (Germany). Anti-mouse IgG+IgM (H+L)-AP conjugate, ECF substrate and Cy5-labelled anti-mouse IgG (H+L) were the products of AP Biotech (UK). α-Galactosidase was obtained from Boehringer Mannheim (Germany) and mouse monoclonal antibodies B16 (anti-B_{tri}) and A9 (anti-A_{tri}) were from Allrussian Hematology Research Center (Russia).

Preparation of glycochips by immobilization of biotinylated glycoconjugates on the surface of sensing elements.

Streptavidin coated chips XNA on Gold™ were purchased from Interactiva, Germany or prepared according to Example 1 of WO97/49989 and incubated with the solutions of biotinylated glycoconjugates of types (**2**) and (**3**) in PBS (5mM - 150nM, 1μl per spot) for 60 min at 37°C. After incubation glycochips formed were washed twice with PBS containing 0.1% Tween (washing buffer).

Revealing of carbohydrate ligands on the surface of glycochips.

Example 1. Glycochips were incubated with excess volume of A9 mAbs (anti-A_{tri}) (dilution 1:10 in PBS with 0.3% BSA) for 90 min at 37°C and washed twice with washing buffer. To visualize bounded antibodies glycochips were incubated with Cy5-labelled anti-mouse antibodies (1:500) for 60 min at 37°C and washed twice with washing buffer. Fluorescence was measured by Fluorescent Image Analyzer FLA-200 (Fujifilm).

Example 2. Glycochips were incubated with excess volume of A9 mAbs (anti-A_{tri}) (dilution 1:10 in PBS with 0.3% BSA) for 90 min at 37°C and washed twice with

washing buffer. To visualize bounded antibodies glycochips were incubated with anti-mouse antibodies-AP conjugate(1:10 000 in PBS with 0.3% BSA) for 60 min at 37°C and washed twice with washing buffer. Then 1μl of ECF substrate was added per spot and after incubation for 5 min the fluorescence was measured by Fluorescent Image Analyzer FLA-200 (Fujifilm).

Treatment of carbohydrate ligands with α-galactosidase on the surface of glycochip.

α-Galactosidase (1:10 in PBS with 0.3% BSA; 1μl per spot) was added onto glycochip (control spots were incubated with 1μl of PBS). After incubation for 60 min at 37°C the glycochips were washed twice with washing buffer and subjected to visualization by using of the combination of mouse monoclonal B16 antibodies (anti-B_{tri}) and anti-mouse-AP conjugate.

Abbreviations

PBS- phosphate buffer solution, pH7.4; BSA - bovine serum albumin; AP - alkaline phosphatase; mAbs - monoclonal antibodies.

A_{tri} GalNAcα1-3(Fucα1-2)Galβ; B_{tri} Galα1-3(Fucα1-2)Galβ; H_{di} Fucα1-2Galβ

Claims

1. An array of discrete sensing elements of glycans (glycoarray) wherein glycans of the type G-Y-X (1) are immobilized on a solid support and wherein
X is an attachment group for immobilization on the solid support;
Y is a linker group;
G is a glycan moiety comprised of at least one or more saccharides.

2. The glycoarray of claim 1, wherein the solid support is coated with the sensing surface of a self-assembling monolayer.

3. The glycoarray of claim 2, wherein the self-assembling monolayer consists of hydrocarbon chains, preferably nonbranched, optionally interrupted by hetero atoms, and of a length exceeding 10 atoms, preferably containing 16-30 atoms.

4. The glycoarray of any one of claims 1 to 3, wherein the linker group is a monomeric, oligomeric or polymer-based spacer for presentation of carbohydrate display, optionally containing a fragment which is cleavable by chemical and/or physical methods.

5. The glycoarray of any one of claims 1 to 4, wherein the glycan moiety is a moiety of a natural or chemically or enzymatically prepared carbohydrate molecule selected from the group consisting of monosaccharide, oligosaccharide, polysaccharide, glycolipid, glycoprotein, glycocompounds of any type with natural, modified natural or non-natural structure mimetics of carbohydrates and any of their combinations.

6. The glycoarray of any one of claims 1 to 5, wherein said glycan moiety is modified by use of chemical, physical and/or enzymatic techniques.

7. The glycoarray of any one of claims 1 to 6, wherein said solid support is silicon, glass, mica, plastic, gallium arsenide, platinum, silver, copper, gold or a combination of any one thereof.

8. The glycoarray of any one of claims 2 to 7, wherein said sensing surface comprises biotin and streptavidin or derivatives thereof.

9. The glycoarray of claim 8, wherein streptavidin forms a nearly 100% surface coverage which prevents direct interaction of the sample with the surface below the sensing surface.

10. The glycoarray of any one of claims 1 to 9, wherein X is biotin or a derivative thereof.

11. The glycoarray of any one of claims 1 to 10, wherein said sensing elements have a density between 5 and 60 fmol/cm^2 on the sensing surface.

12. A method of producing a glycoarray of any one of claims 1 to 11, comprising immobilization of a glycan as defined in claim 1 on a streptavidin coated sensing surface via biotin or a derivative thereof.

13. A method for discriminating complex biological samples using a glycoarray of any one of claims 1 to 11 preferably in which constituents bound to the glycoarray are determined by label or non-label detection systems.

14. The method of claim 13, wherein said non-label detection system comprises measuring the increase in mass or thickness on the surface of the glycoarray.

15. The method of claim 13 or 14, wherein said surface mass increase detection techniques are selected from the group consisting of quartz crystal microbalances, optoaucostics, reflectometry, ellipsometry, SAW, surface plasmon resonance, mass spectrometry, capacitive and amperometric techniques.

16. The method of any one of claims 13 to 15, wherein said detection is performed in imaging mode with a CCD camera or a scanning based device.

17. A method of diagnosing a disease, comprising contacting a sample from a subject with a glycoarray of any one of claims 1 to 11.

18. The method of claim 17 further comprising a method of any one of claims 13 to 16.

19. The method of claim 17 or 18, wherein said sample is a human or animal, tissue or bodily fluid, selected from the group consisting of blood, serum, urine, milk, sweat, exhaled air, skin, bone marrow, cerebrospinal fluid, synovial fluid, amniotic fluid and lymphatic fluid.

20. The method of any one of claims 17 to 19, wherein said disease is selected from the group consisting of genetic disorders, autoimmune diseases, arthritis, infectious diseases, cancer, heart disease, drug abuse HIV, BSE and lung disease.

21. A method of diagnosing the general state of health, said method comprising the method of any one of claims 13 to 20, wherein a signal pattern is generated which is diagnostic of a particular state of health, said sample is a patient sample and said standard is the pattern present in a representative part of the population.

22. The method of claim 21, wherein said general state of health is selected from common mild ailments and/or health conditions with diffuse symptoms, consisting of high blood pressure, pregnancy, common colds, injuries, inflammatory reactions, mild immune suppression, doping, altitude sickness, space sickness chronic fatigue syndrome, and effects of low level toxic chemical or radiation exposure, menstrual cycles and subclinical infections.

23. A method of identifying an organism, said method comprising the method of any one of claims 13 to 16, wherein a signal pattern is generated that is unique to a particular organism, said sample is a biological sample from a particular organism and said standard is the pattern normally found in that organism.

24. A kit comprising a glycoarray of any one of claims 1 to 11 or produced by the method of claim 12, and optionally suitable means for detection as defined in any one of claims 13 to 16.

25. Use of a glycoarray of any one of claims 1 to 11, or produced by the method of claim 12 for a diagnostic assay.

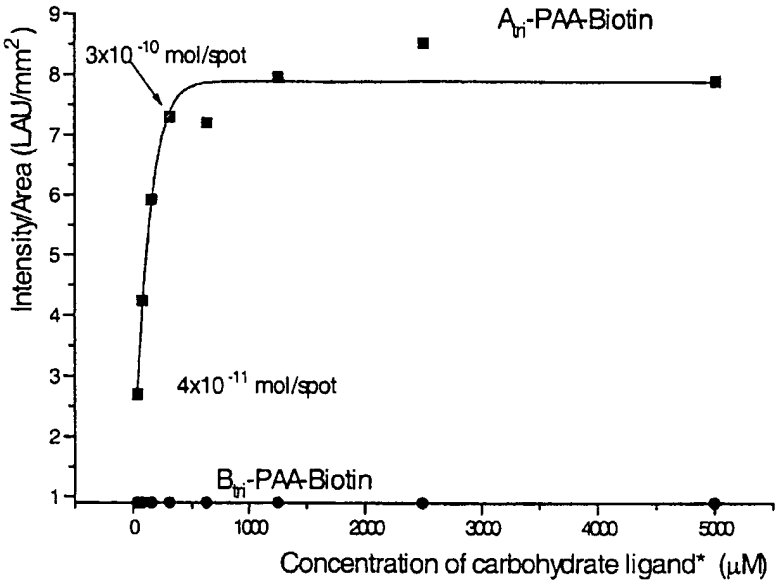

Intensity/Area (LAU/mm²)
Concentration of carbohydrate ligand* (µM)
A$_{tri}$-PAA-Biotin
B$_{tri}$-PAA-Biotin
3x10^{-10} mol/spot
4x10^{-11} mol/spot
1
2
3
4
5
6
7
8
9
0
1000
2000
3000
4000
5000

Figure 1

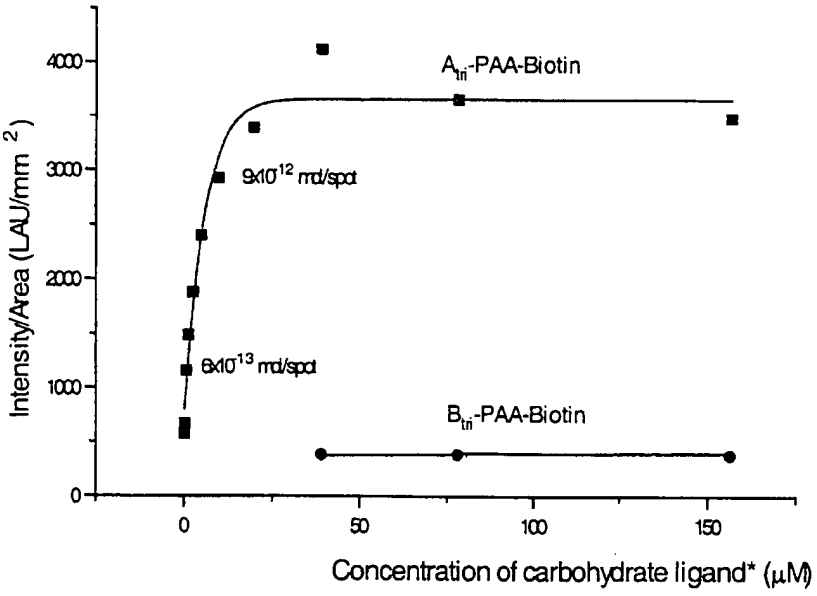

Intensity/Area (LAU/mm 2)
4000
3000
2000
1000
0
0
50
100
150
Concentration of carbohydrate ligand* (μM)
A tri-PAA-Biotin
9x10 12 mol/spot
6x10 13 mol/spot
B tri-PAA-Biotin

Figure 2

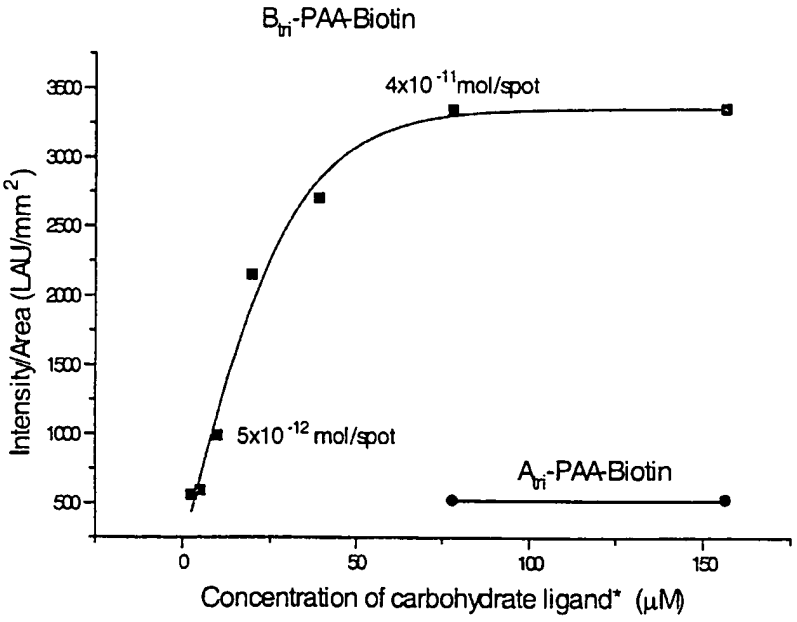

B$_{tri}$-PAA-Biotin
4x10^{-11}mol/spot
5x10^{-12} mol/spot
A$_{tri}$-PAA-Biotin
Intensity/Area (LAU/mm^2)
3500
3000
2500
2000
1500
1000
500
0
50
100
150
Concentration of carbohydrate ligand* (µM)

Figure 3

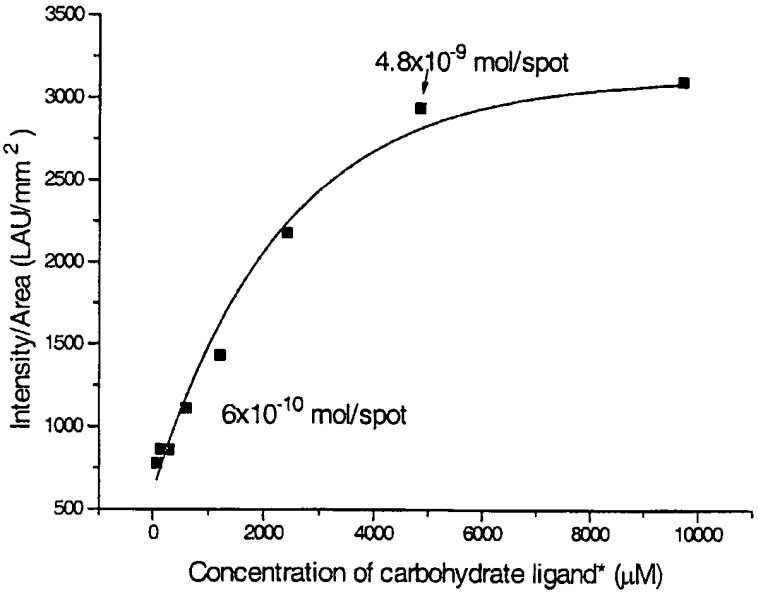

3500
3000
2500
2000
1500
1000
500
Intensity/Area (LAU/mm 2)
4.8x10-9 mol/spot
6x10-10 mol/spot
0
2000
4000
6000
8000
10000
Concentration of carbohydrate ligand* (µM)

Figure 4

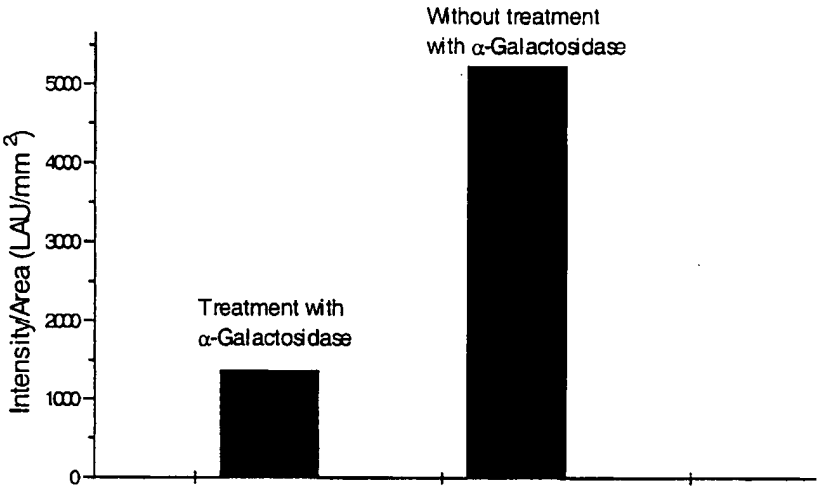

Without treatment
with α-Galactosidase
Treatment with
α-Galactosidase
Intensity/Area (LAU/mm 2)
5000
4000
3000
2000
1000
0

Figure 5